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Conformational Change and Activity Enhancement of Rabbit Muscle Lactate Dehydrogenase Induced by Polyethyleneimine

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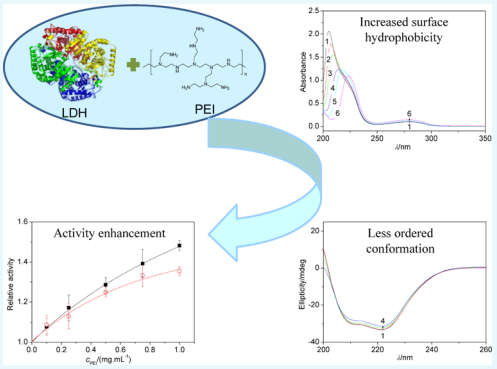
ABSTRACT: For a better understanding on the interaction between polyethyleneimine (PEI) and proteins, spectroscopic studies including UV−vis absorption, resonance Rayleigh scattering, fluorescence, and circular dichroism were conducted to reveal the conformational change of rabbit muscle lactate dehydrogenase (rmLDH) and related to the bioactivity of the enzyme. Regardless of the electrostatic repulsion, PEI could bind on the surface of rmLDH, a basic protein, via hydrogen binding of the dense amine groups and hydrophobic interaction of methyl groups. The competitive binding by PEI led to a reduction of the binding efficiency of rmLDH toward β-nicotinamide adenine dinucleotide, the coenzyme, and sodium pyruvate, the substrate. However, the complex formation with PEI induced a less ordered conformation and an enhanced surface hydrophobicity of rmLDH, facilitating the turnover of the enzyme and generally resulting in an increased activity. PEI of higher molecular weight was more efficient to induce alteration in the conformation and catalytic activity of the enzyme.



1. INTRODUCTION

The wide application of biomaterials in the past decades played important roles to improve human health and brought about revolution in the fields of medicine and biotechnology. Before the application in the living system, the safety of biomaterials must be strictly evaluated due to the fact that they will inevitably contact various biomacromolecules, cells, tissues, and organs. Polyethyleneimine (PEI) was considered as the "golden standard" for polymeric gene delivery systems due to the capability to condense DNA and RNA into nanoparticles and transfer into cells via endocytosis.[1,2] However, evidences showed that PEI also induced somewhat cytotoxicity, for which the molecular basis was far from understood, and the knowledge about the mechanism of the interaction between PEI and biomacromolecules is still limited.[3,4] Due to the protonation of amine groups on the polymer chain of PEI, electrostatic interaction was proposed to dominate the complex formation with proteins.[5] However, it was reported that PEI increased the stability of porcine muscle lactate dehydrogenase[6] (LDH, a basic protein with pI ∼ 8.2) and glucose dehydrogenase[7] (an acid protein with pI ∼ 6.0). Previous work from our group suggested that hydrophobic interaction and hydrogen bond dominated the surface binding of PEI on horseradish peroxidase (HRP, a neutral protein of pI ∼ 7.0) and pig heart LDH (phLDH, an acid protein of pI ∼ 5.6),[6] leading to a more compact conformation.[8,9]

It was logical to question that whether the electrostatic interaction also contributed to the interaction of PEI with proteins. LDH consists of a family of five isoenzymes which is composed of two types of subunit (H and M). Due to the high sequence homology of H- and M-type subunits, the heart and muscle LDH were similar in their catalytic function and molecular shape.[10] However, as a result of the difference in the number of acid and basic amino acid residues and the exposure to solvent, heart and muscle LDH exhibited opposite surface charge, which was considered to be responsible for the great difference in the catalytic parameters, isoelectric points, and thermodynamic stability of the two enzymes and thus were good models for exploring the underlying mechanism of PEI interaction with proteins.[5,6]

As an extension of the previous work,[9] the conformational change of rabbit muscle LDH (rmLDH, a basic protein with pI ∼ 8.2)[6] in the solutions containing PEI of typical molecular weights 25,000 and 1800 Da (labeled as PEI25k and PEI1.8k, respectively.) was investigated by UV−vis absorption, resonance Rayleigh scattering (RRS), fluorescence, and circular dichroism (CD). Enzymatic activity of rmLDH was also monitored by the catalytic reduction of sodium pyruvate to lactate using β-nicotinamide adenine dinucleotide (NADH) as the coenzyme. A comparison was made to the effects of PEI on

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the conformation and biofunction of rmLDH and phLDH,[9] with the purpose to have a better understanding on the mechanism of PEI−protein interaction.

2. RESULTS AND DISCUSSION

2.1. UV−Vis Absorption. Absorption spectra of rmLDH in PEI-containing phosphate-buffered solution (PBS) are presented in Figure 1. In PBS, a strong absorption of amide

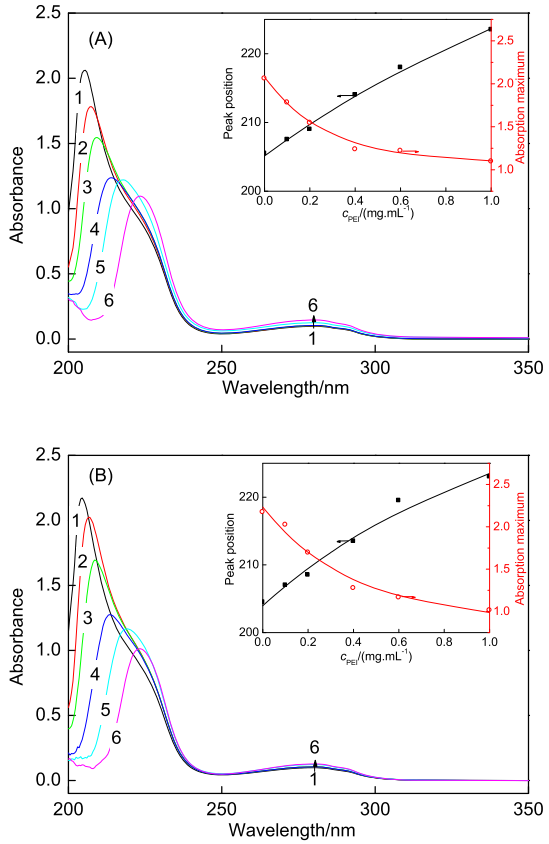

Figure 1. Absorption spectra of rmLDH in PBS containing PEI25k (A) and PEI1.8k (B). Inset: PEI concentration dependence of maximum absorption and peak position. c_{rmLDH} = 0.5 μM, c_{PEI}: (1) 0, (2) 0.1, (3) 0.2, (4) 0.4, (5) 0.6, and (6) 1.0 mg·mL^{-1}.

groups and a weak absorption of aromatic residues were observed around 280 and 208 nm, respectively. The absorptions were usually related to the framework of the peptide chain and the microenvironment around aromatic residues and thus an indication of the conformation change of proteins.[11,12] As shown in Figure 1, the absorption maximum of amide groups decreased with increasing PEI concentration, accompanying a blue shift of the peak position, indicating a possible alteration in the secondary structure of rmLDH due to the interaction with PEI.[11,12] PEI had little effects on the peak position of aromatic residue absorption of rmLDH. However, the slight increase in the absorption suggested a perturbation of the tertiary structure of the enzyme, leading to an increased exposure of aromatic residues.

2.2. RRS. RRS is a useful tool to investigate the interaction of biomacromolecules.[13,14] To confirm the interaction between PEI and rmLDH, RRS was conducted to PEI solution in the absence and presence of rmLDH. Weak scattering with two peaks around 310 and 355 nm was observed for PBS buffer. Addition of PEI had little influence on the scattering patterns of the buffer; however, it led to an increase in the intensity (Figure 2). Similar results were observed for rmLDH solutions

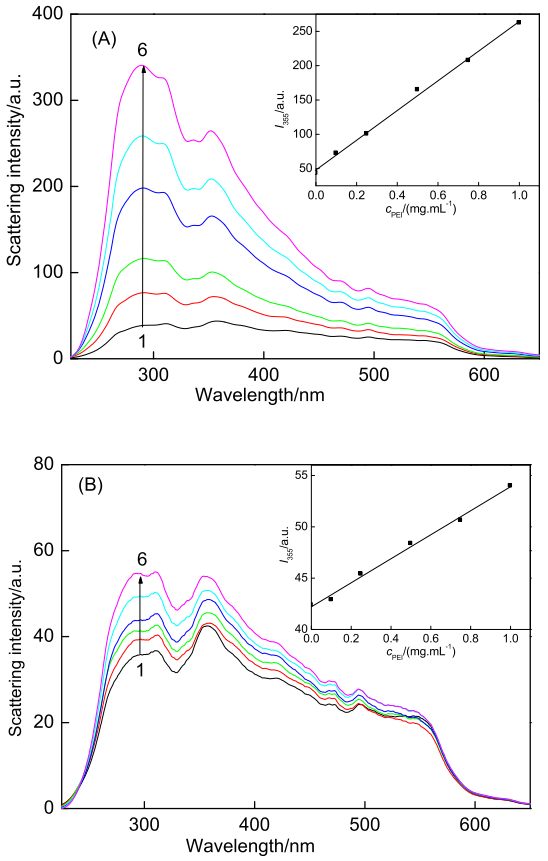

Figure 2. RRS pattern of PBS containing PEI25k (A) and PEI1.8k (B). Inset: Scattering intensity of the solutions at 355 nm. c_{PEI}: (1) 0, (2) 0.1, (3) 0.25, (4) 0.5, (5) 0.75, and (6) 1.0 mg·mL^{-1}.

with and without PEI, except a decrease in scattering around 280 nm, resulting from the absorption of aromatic residues of the enzyme (Figure 3). To avoid the influence of the absorption of rmLDH, the scattering intensity at 355 nm was selected for both solutions and is shown in Figures 2 and 3.

RRS was sensitive to the particle size in the solution. Based on the Rayleigh principle, the intensity of scattering at a given wavelength was expected to be proportional to the sixth power of the size of the particle and its concentration in the solution.[13,14] The linear increase in the scattering of PEI solution indicated that no aggregation of PEI occurred in the solutions (Figure 2). However, the scattering of PEI−rmLDH solution leveled off with increased PEI concentration (Figure 3), suggesting the interaction with PEI and a possible conformational change of the enzyme, which was also supported by CD as follows.

2.3. CD Spectra. CD spectra of rmLDH in PBS containing PEI of typical concentration were obtained. In PBS, the CD spectra of rmLDH was characterized by two negative bands around 208 and 222 nm, which was attributed to the α-helical structure (Figure S1 in the Supporting Information).[15,16] The secondary structure estimation by the CDSSTR method[17] indicated 40% α-helices, 12% β-sheets, 14% β-turn, and 34% random coil for rmLDH in the absence of PEI, approximate to those of pgLDH from our previous work.[9] PEI addition

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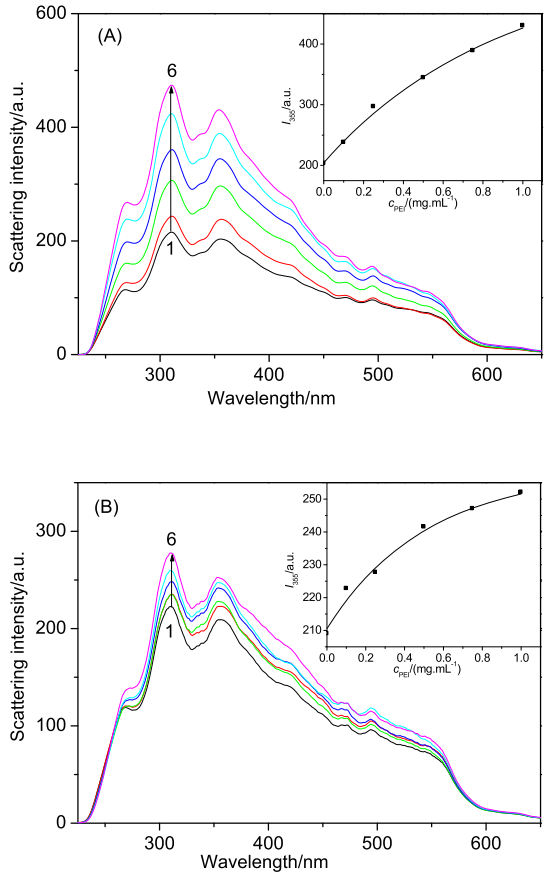

Figure 3. RRS patterns of rmLDH solutions in PBS containing PEI25k (A) and PEI1.8k (B). Inset: Scattering intensity of the solution at 355 nm. (c_{rmLDH} = 1 μM, c_{PEI}: (1) 0, (2) 0.1, (3) 0.25, (4) 0.5, (5) 0.75, and (6) 1.0 mg·mL^{-1}).

resulted in a transition from α-helix to β-sheet and random coil (Table 1), indicating a less ordered conformation of rmLDH. The result was opposite to that of phLDH, suggesting a difference in the interaction with PEI.

Table 1. Secondary Structure Content of rmLDH in the Absence and Presence of PEI[a]

medium	α-helix/%	β-sheet/%	β-turn/%	random coil/%
PBS	40	12	14	34
PEI1.8k/PBS	35	15	14	36
PEI25k/PBS	33	16	14	37

[a] c_{PEI} = 1.0 mg·mL^{-1}.

Due to the alternative distribution of hydrophobic methyl groups and hydrophilic amine groups along the polymer chain, PEI was difficult to penetrate into the hydrophobic core of proteins and a surface binding was preferred.[18,19] PEI was found to bind onto the surface of HRP, a neutral protein with pI ~ 7.0, via hydrophobic interaction and hydrogen bond and induced a more compact conformation.[8] Hydrophobic interaction and hydrogen bond were also found between PEI and pgLDH[10] and was expected to be involved in the interaction with rmLDH due to the high-sequence homology of the two enzymes.[6] Thus, the difference in the conformation change of pgLDH and rmLDH may have resulted from electrostatic interaction. Electrostatic calculation showed that the heart LDH was richer in negative electrostatic potential patches on the surface due to more acidic groups and less basic groups, opposite to the muscle LDH.[6] The results from this work indicated that electrostatic interaction also played an important role in the conformation stability of proteins. An intimate contact with PEI due to the electrostatic attraction favored a more compact conformation of proteins.

2.4. Intrinsic Fluorescence. The intrinsic fluorescence of proteins, specially originating from tryptophan residues, is sensitive to the polarity of the local environment and thus a common tool to probe the conformational change of proteins. In this work, fluorescence emission of tryptophan was collected at λ_{ex} = 295 nm to avoid the overlap with that of tyrosine and the resonance energy transfer to tryptophan (Trp).[20] Tryptophan of rmLDH displayed a fluorescence emission around 340 nm in PBS. Addition of PEI had little influence on the peak position; however, it led to an increase in the intensity (Figure 4), which was quite different from the fluorescence quenching of phLDH induced by the surface binding of PEI.[9]

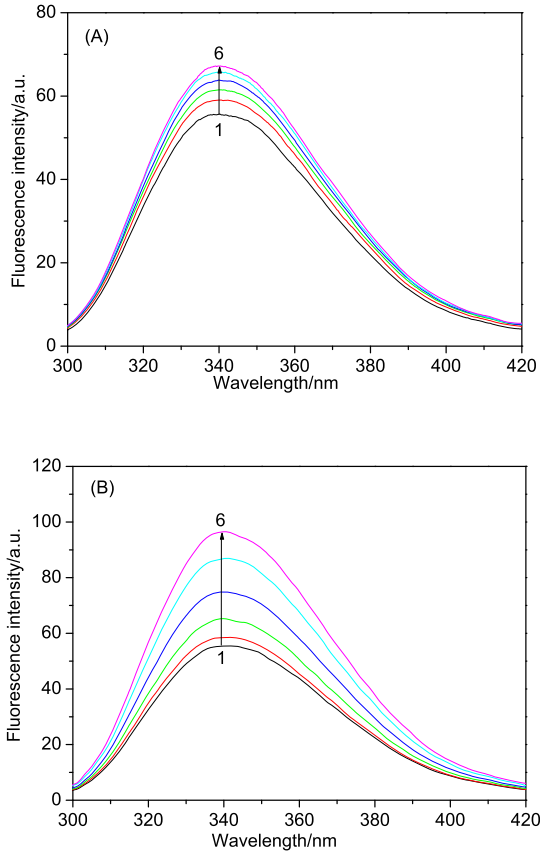

Figure 4. Fluorescence emission of rmLDH in PBS containing PEI25k (A) and PEI1.8k (B). λ_{ex} = 295 nm, c_{rmLDH} = 1 μM, c_{PEI}: (1) 0, (2) 0.1, (3) 0.25, (4) 0.5, (5) 0.75, and (6) 1.0 mg·mL^{-1}.

As illustrated in the previous work, the binding of PEI at the surface of proteins usually resulted in preferential fluorescence quenching of tryptophan residues located outside of the hydrophobic core.[21,22] Among the tryptophans in the subunit of phLDH, Trp323 was exposed and Trp150 partly exposed to the solvent, and the rest (Trp190, 203, 225 and 248) was buried in the internal of the protein.[23] As suggested by Burstein, the emission of tryptophan appeared at 331 nm in an apolar environment and moved to 351 nm in water.[24] The peak position at 348 nm indicated that the fluorescence

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emission was dominated by tryptophan residues located at the surface of phLDH.[9] The preferential fluorescence quenching of tryptophan residues exposed was responsible for the blue shift of the fluorescence emission of pgLDH,[9] just as bovine serum albumin[21] and lysozyme.[22] In the case of rmLDH, there are five tryptophans (Trp148, 188, 201, 227, and 250) in each of the four M-type subunits.[10] The fluorescence emission maximum that appeared around 340 nm suggested the lack of tryptophan residues located at the surface of rmLDH. Thus, no fluorescence quenching was observed for rmLDH due to PEI binding (Figure 4), just as human serum albumin.[25] On the contrary, an enhancement of tryptophan emission was observed, maybe due to the increased absorption of aromatic residues (Figure 1) and the weakened collisional quenching by water molecules as a result of PEI binding on the surface of rmLDH. As compared to PEI25k, the enhancement of tryptophan emission was more evident in the presence of PEI1.8k (Figure 4), which was consistent with the fact that the smaller size of lower molecular weight PEI facilitated surface binding in a flat conformation.

2.5. ANS Fluorescence. To reveal the possible change in the tertiary structure of rmLDH, the fluorescence emission of 8-anilino-1-naphthalenesulfonic acid (ANS) was obtained. ANS displayed a weak fluorescence emission around 525 nm in PBS. An increase in ANS fluorescence emission and an accompanying blue shift were observed upon addition of PEI, indicating the weak hydrophobic nature of PEI and the binding with ANS[26] (Figure S2 in the Supporting Information). Similar results were obtained in the presence of rmLDH (Figure S3 in the Supporting Information). However, the enhancement of ANS fluorescence was much less evident than that when ANS was bound into the hydrophobic core of bovine serum albumin,[21] indicating that ANS was bound on the surface of rmLDH.

Consistent with the results for pgLDH,[9] the fluorescence emission of ANS increased with increasing PEI concentration in the solutions with and without rmLDH (Figure 5). Due to the competitive binding by PEI, the fluorescence emission of ANS in ANS−PEI−rmLDH solutions was expected to approach that in the absence of the enzyme. However, the difference in fluorescence intensity first increased and then decreased with increasing PEI concentration in the solutions (Figure 5), suggesting an increase in the surface hydrophobicity of rmLDH due to the interaction with PEI, which was consistent with the increased exposure of aromatic residues illustrated by UV−vis absorption (Figure 1).

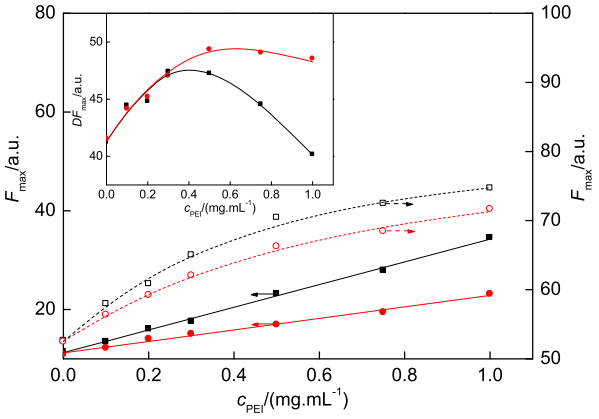

Figure 5. Fluorescence emission maximum of ANS in PEI-containing solutions in the absence (solid line) and presence (dash line) of rmLDH. Inset: Difference of ANS fluorescence emission maximum in the absence and presence of rmLDH. (■□) PEI25k, (●○) PEI1.8k, λ_{ex} = 360 nm, c_{rmLDH}, c_{ANS} = 4 μM.

PEI is a polymer carrying large quantity of amine groups prone to protonation. The decrease in protonation with increasing molecular weight of PEI, as illustrated by zeta-potential measurement[9] and pK_a value determination,[27] implied an enhanced hydrophobic interaction with ANS, agreeing well with the fact that the increase and blue shift of peak emission of ANS was more evident in the presence of PEI25k as compared to PEI1.8k (Figure S2 in the Supporting Information). As a consequence, the difference in ANS fluorescence in ANS−PEI−rmLDH and ANS−PEI solutions reached a maximum at a lower concentration of PEI25k (Figure 5).

2.6. Activity Assay and Kinetics. Enzymatic activity of rmLDH was monitored by the reduction of sodium pyruvate. Opposite to pgLDH,[9] the activity of rmLDH increased with increasing PEI concentration in the range studied. In the solution containing 1.0 mg·mL^{-1} PEI25k and PEI1.8k, the increase in the activity of rmLDH was about 48 and 35%, respectively (Figure 6).

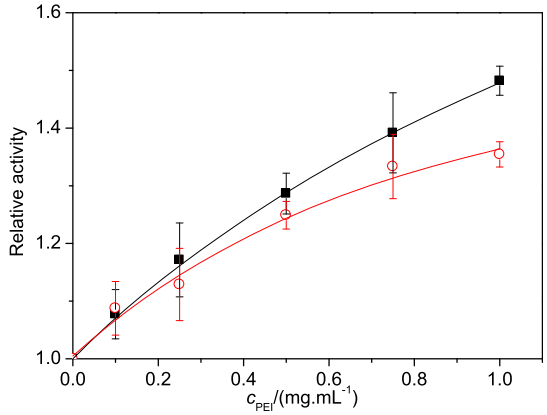

Figure 6. Relative activity of rmLDH in PEI-containing solutions. (■) PEI25k and (○) PEI1.8k.

Steady-state kinetic studies were carried out. The affinity constant K_m and turnover number k_{cat} were obtained from Michaelis−Menten equation. For the sake of comparison with the activity assay, the initial reaction rate at the NADH concentration of 0.22 mM and the sodium pyruvate concentration of 1.0 mM was calculated. The relative activity of the enzyme was also evaluated by taking the initial reaction rate in PBS as the control (Tables 2 and 3). The increased K_m value indicated a decrease in the affinity of rmLDH toward NADH and sodium pyruvate due to the competitive binding by PEI.[9] However, the increase in the turnover of the enzyme resulted in an enhanced activity of rmLDH, opposite to pgLDH.[9] It was found that PEI of higher molecular weight (PEI25k) was more efficient to increase the initial rate of the reaction (Tables 2 and 3), agreeing well with the catalytic activity assay (Figure 6).

It was well known that the conformational change of active sites was fundamental for the catalysis by LDH.[28] The reduction of sodium pyruvate was initiated by the binding of substrate to the binary complex of LDH·NADH.[29] Molecular dynamics simulation showed that the binding of substrate

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Table 2. Kinetic Parameters of Sodium Pyruvate Reduction Catalyzed by rmLDH Using Constant Sodium Pyruvate Concentration and Varying NADH Concentrations

medium[b]	$K_m \times 10^3$ (mM)[c]	$k_{cat} \times 10^{-3}$ (min^{-1})	$v \times 10^3$ ($mM \cdot min^{-1}$)[d]	relative activity
PBS	8.3(1.1)	24.3(0.3)	8.0	1
PEI1.8k/PBS	28.4(4.1)[a]	34.2(1.4)[a]	11.1	1.38
PEI25k/PBS	26.1(3.5)[a]	39.3(1.4)[a]	12.7	1.58

[a]$P < 0.05$, one-way analysis of variance (ANOVA) analysis. [b]$c_{PEI} = 1.0$ $mg \cdot mL^{-1}$. [c]Values in brackets represent the standard deviation. [d]Reaction rate at NADH concentration of 0.22 mM and sodium pyruvate concentration of 1.0 mM.

Table 3. Kinetic Parameters of Sodium Pyruvate Reduction Catalyzed by rmLDH Using Constant NADH Concentration and Varying Sodium Pyruvate Concentrations

medium[b]	K_m (mM)[c]	$k_{cat} \times 10^{-3}$ (min^{-1})	$v \times 10^3$ ($mM \cdot min^{-1}$)[d]	relative activity
PBS	1.4(0.2)	66.1(8.1)	9.1	1
PEI1.8k/PBS	3.6(0.3)[a]	170.6(17.9)[a]	12.2	1.35
PEI25k/PBS	6.8(0.25)[a]	327.7(27.3)[a]	14.1	1.55

[a]$P < 0.05$, ANOVA analysis. [b]$c_{PEI} = 1.0$ $mg \cdot mL^{-1}$. [c]Values in brackets represent the standard deviation. [d]Reaction rate at NADH concentration of 0.22 mM and sodium pyruvate concentration of 1.0 mM.

induced a movement of a face loop of peptide chain, the so-called "mobile loop," to the entrance, carrying the key catalytic residue Arg109 into the active site. Consequent conformation transition of the subdomain around the substrate and NADH brought them in close contact and in a proper geometry for the transfer of hydride ion from the C4 carbon of the nicotinamide group of NADH to the C2 carbon of pyruvate. The rate-limiting step in the turnover of LDH was the closure of the mobile loop over the substrate binding pocket, rather than the chemical hydride transfer.[30,31] The hurdle to the motion of the mobile loop by PEI bound on the surface was suggested to be responsible for the decrease in the turnover number of pgLDH.[9] Similar results were also expected for rmLDH since PEI binding is nonspecific. However, PEI binding led to an increase in the turnover of rmLDH (Tables 2 and 3), suggesting that the perturbation of catalysis by LDH may be related to the conformational change of the enzyme. As illustrated by absorption (Figure 1) and CD analysis (Figure 4), PEI induced a less compact conformation of rmLDH, opposite to pgLDH.[9] The results indicated that a less ordered conformation of LDH was helpful for the motion of the mobile loop and thus the turnover of the enzyme and vice versa. Spectroscopic studies showed that *N*-methylimidazolium-based ionic liquids induced a decrease in the intensity and a blue shift of the peak position of tryptophan fluorescence and an enhanced negative Cotton effect of far-UV CD, indicating a more compact conformation of rmLDH. It was found that *N*-methylimidazolium-based ionic liquids displayed inhibitory effects on the activity of rmLDH,[32] which was similar to the results of pgLDH in the presence of PEI.

3. CONCLUSIONS

Regardless of the electrostatic repulsion, PEI could bind on the surface of rmLDH, a basic protein, via hydrogen binding and hydrophobic interaction with the amine groups and the methyl groups, respectively. The complex formation between rmLDH and PEI resulted in a less ordered conformation and an enhanced surface hydrophobicity of the enzyme. The competitive binding by PEI led to a reduction in the binding efficiency of rmLDH toward the coenzyme and the substrate. However, the conformational change of rmLDH facilitated the turnover of the enzyme and generally resulted in an increased activity, opposite to phLDH, the enzyme of high sequence homology but richer in negative potential patches on the surface. The alteration in the conformation and bioactivity of rmLDH was more evident due to the interaction with PEI of higher molecular weight.

4. MATERIALS AND METHODS

4.1. Materials. Branched PEI, LDH (from rabbit muscle, type XI, lyophilized powder, 600−1200 $U \cdot mg^{-1}$), sodium pyruvate (>99%), NADH (>98%), and ANS (≥97%, high-performance liquid chromatography) were used as received from Sigma-Aldrich (USA). For the sake of comparison with our previous work,[9] PBS at pH 7.4 was used throughout the experiment, approximate to physiological conditions in vivo.

Stock solutions of PEI, sodium pyruvate, NADH, and ANS were prepared by dissolving the products in PBS and adjusted to pH 7.4 using HCl and NaOH solutions. Concentrations of NADH and ANS were determined based on the absorption at 340 nm and 350 nm, at which the extinction coefficient was 6220 and 5000 $M^{-1} \cdot cm^{-1}$, respectively.[26,29] Lyophilized rmLDH was dissolved in PBS and centrifuged to remove insoluble impurities. Enzyme concentration was determined based on the absorption at 280 nm with an extinction coefficient of 1.96×10^5 $M^{-1} \cdot cm^{-1}$.[33] The stock solutions were diluted to the desired concentration and incubated at 25 °C for 30 min before spectra analysis.

4.2. Methods. *4.2.1. UV−Vis Absorption.* UV−vis absorption of rmLDH from 200 to 350 nm was collected using an UV-2501PC UV−vis spectrophotometer (Shimadzu, Japan) at 25 °C. To eliminate the influence of PEI absorption in the far-UV region, PBS containing PEI of the same molecular weight and of the same concentration was used as reference.

4.2.2. RRS. RRS spectra of PEI and PEI−rmLDH solutions were obtained on a RF5301PC spectrofluorimeter (Shimadzu, Japan) at 25 °C by scanning synchronously the excitation and emission monochromators with $\Delta\lambda = \lambda_{ex} - \lambda_{em} = 0$.

4.2.3. CD Measurement. CD performance was conducted on a MOS-450 spectropolarimeter (Bio-Logic, France) in nitrogen atmosphere using a quartz cell of 1 mm path length. Far-UV spectra of rmLDH in PEI-containing solutions, from 190 to 260 nm, were collected at 0.5 nm intervals with an integration time of 1 s. The possible influence of PEI was eliminated by a blank measurement for the medium under the same conditions.

4.2.4. Fluorescence. Fluorescence emission was performed on a RF6000 spectrofluorimeter (Shimadzu, Japan) at 25 °C. The fluorescence emission of rmLDH was excited at 295 nm and recorded from 300 to 450 nm, and that of ANS was excited at 360 nm and recorded from 370 to 700 nm.

4.2.5. Activity Assay. Catalytic activity of rmLDH was assayed by modified Worthington method, as described in previous work.[9] Briefly, both the enzyme solution containing 0.01 μM rmLDH and the reaction mixture containing 1.0 mM sodium pyruvate and 0.22 mM NADH were prepared in PBS containing PEI of the same concentration. After incubation at

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25 °C for 30 min, 0.1 mL of enzyme solution was blended with 2.9 mL of reaction mixture in a 1 cm × 1 cm quartz cell and kept at 25 °C. The rate of reaction was monitored by the absorption of NADH at 340 nm. The activity of rmLDH was evaluated by the initial consumption of NADH in the first 5 min.[34] The experiment was carried out at least three times. The relative activity was calculated by using the value in PBS as a control.

4.2.6. Catalytic Kinetics. Steady-state kinetic experiments were conducted following the same procedure as activity assay, except using varying concentrations of NADH (0.05−0.1 mM) or sodium pyruvate (0.4−1 mM). The initial rate of the reaction, ν, was fitted to Michaelis−Menten equation to obtain the affinity constant K_m and turnover number k_{cat}.

$$\nu = k_{cat}[E]_0[S]/(K_m + [S]) \quad (1)$$

whereas $[E]_0$ is the molarity of enzyme and $[S]$ is the concentration of sodium pyruvate or NADH. The performance was carried out at least three times.

4.2.7. Statistical Analysis. The results of activity assay and kinetics were statistically analyzed using one-way ANOVA method. $P < 0.05$ was considered significant compared with the control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00562.

Far-UV CD spectra of rmLDH in PEI-containing PBS; fluorescence emission of ANS in PEI-containing PBS; and fluorescence emission of ANS in PEI-containing PBS in the presence of rmLDH (PDF)

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Notes

The authors declare no competing financial interest.

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